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Effect of Salivary Factors on the Susceptibility of Hydroxyapatite to Early Erosion

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Key Words

Dental erosion • Hydroxyapatite • Pellicle • Saliva • Susceptibility

Abstract

Objective: Salivary pellicle is known to reduce the erosion of enamel and differences in the level of protection exist between individual saliva sources, but which parameters or components are important is not known. The focus of this study was to investigate the relationship between saliva parameters and early erosion of hydroxyapatite (HAp) with an in situ grown saliva film. **Methods:** Twenty-eight volunteers carried two HAp and one porcelain discs in their buccal sulcus for 1.5 h. Next, the discs covered with pellicle and the attached saliva film were exposed extraorally to 50 mM (pH = 3) citric acid for 2 min and unstimulated and stimulated saliva was collected. Calcium loss from HAp after erosive challenge was measured, corrected for calcium loss from pellicle on porcelain discs and averaged. Several salivary parameters were analysed. Pearson's linear correlation and multiple regression analysis were used to study the relation between saliva parameters and HAp erosion. **Results:** Significant correlations were found between HAp erosion and the concen-

tration of phosphorus in unstimulated saliva ($r = 0.40$, $p = 0.03$) and between HAp erosion and the concentration of sodium ($r = -0.40$, $p = 0.03$), chloride ($r = -0.47$, $p = 0.01$), phosphorus ($r = 0.45$, $p = 0.01$) and flow ($r = -0.39$, $p = 0.04$) of stimulated saliva. Multivariate analysis revealed a significant role in the HAp erosion for sodium, urea, total protein, albumin, pH and flow of unstimulated saliva and for sodium, potassium, urea, and phosphorus of stimulated saliva. **Conclusions:** Several salivary parameters are associated with the susceptibility of HAp to erosion.

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Wide variation between individuals has been found regarding their susceptibility to develop dental erosion [O'Sullivan and Curzon, 2000; Vieira et al., 2007]. Also in in vitro research it was found that saliva from different donors affords different levels of protection against erosion [Wetton et al., 2007] and in an in situ study it was found that the difference between high and low eroders can be up to 10-fold [Hughes et al., 1999b]. Moreover, results of in vitro studies investigating the erosive potential of soft drinks showed losses of enamel many orders of magnitude greater than recorded on specimens in situ

[West et al., 1998; Hughes et al., 1999a]. In all these phenomena saliva may play an important role [Hall et al., 1999].

Saliva can theoretically protect against erosion in several ways, but it is unclear how effective its protective capacity is. Saliva can act as a diluting agent for acids and salivary clearance removes the acid gradually via the swallowing process. In addition saliva contains phosphate, protein and bicarbonate buffers and saliva is supersaturated with respect to tooth minerals, such as calcium and phosphate. Moreover, saliva contains a wide array of proteins and some of them might have protective properties. Finally, proteins can protect the teeth by the formation of a salivary pellicle when teeth are exposed to saliva [Siqueira et al., 2007]. This pellicle may act as a barrier for acids [Dawes, 2008]. In hyposalivation, carious destruction and erosive wear are phenomena that occur simultaneously [Jansma et al., 1989; Lajer et al., 2009]. With respect to the development of caries it was found that the salivary pellicle derived from whole saliva plays a preventive role [Featherstone et al., 1993].

It is still unclear which salivary parameters are most important in explaining the differences in susceptibility to erosive wear. We hypothesize that salivary parameters can, at least in part, explain the variability in susceptibility to erosion. Therefore the aim of this study was to investigate the interindividual variation in early erosion of HAp covered with an in situ grown pellicle/salivary film and to relate these to salivary parameters.

Materials and Methods

Subjects, Substrate and Growth of Pellicle

Twenty-eight volunteers with no relevant medical or pharmacotherapy histories (16 females, 12 males) in the age range of 19–59 years were recruited from advertisements displayed in the UMCG. The sample size was based on a previous pilot study (UMCG IRB No. 2007170). On the basis of measured saliva parameters a correlation was found between the concentration of phosphorus in stimulated saliva and the loss of calcium from HAp. With the data from this pilot study sample size was estimated using PS-power software [Dupont and Plummer, 1990]. The σ was calculated with the standard deviation of the independent variable (phosphorus concentration in stimulated saliva, 0.32), the slope of the regression curve (λ , 0.108) and the standard deviation of the dependent variable (loss of calcium from HAp, 1.02). Sample size was calculated with an α of 0.05, a power of 0.8 and a slope of the regression curve of 1. This resulted in an estimated sample size of 24. Only participants with a healthy oral environment (i.e. Dutch Periodontal Screening Index [van der Velden, 2009] score of 1 or lower, no recent caries activity, no erosive wear and no hyposalivation) and with no relevant medical or pharmacotherapy history (American

Society of Anesthesiologists score 1 [Owens et al., 1978]) were allowed to participate. Informed written consent was obtained from all the subjects. The study design was reviewed and approved by the University Medical Center Groningen Investigators Research Board (UMCG IRB No. 2008109).

In every volunteer, two sintered HAp discs (Himed Medical Applications Inc., Old Bethpage, N.Y., USA) and one porcelain disc (IPS Emax Press, Ivoclar Vivadent, Schaan, Principality of Liechtenstein) were placed in the buccal sulcus of the lower jaw in close proximity to the first molar of every volunteer at 9.00 a.m. The discs had a diameter of 8 mm and a thickness of 2 mm. All the HAp discs came from the same batch (batch No. 100406). Before placing the discs in the mouth, the discs were submersed in 15 ml of a standard solution of 50 mM citric acid (pH = 3) for 1 h and rinsed with water to remove any loosely attached or more soluble material. After this exposure the discs were clean and dissolved all in a very homogeneous way [Hemingway et al., 2008].

Eating, drinking, brushing and smoking were not allowed from 1 h before insertion until removal of the samples from the mouth (both the HAp and porcelain samples were 90 min in situ).

Exposure to Citric Acid

The HAp discs covered with pellicle and attached saliva film were removed from the oral cavity and without rinsing were immediately exposed for 2 min to 2 ml of an erosive solution (50 mM citric acid, pH = 3) under agitation (100 rpm) and rinsed with 2 ml of demineralized water. The loss of calcium was determined by atomic absorption spectroscopy as described in a previous publication [Jager et al., 2008]. The porcelain discs were exposed to the erosive solution in a similar fashion to determine calcium loss from pellicle and salivary film only. The loss of calcium from the two HAp discs was averaged and the loss of calcium from the pellicle from the porcelain disc was deducted from this value to correct for the extra calcium measured coming from the pellicle or the salivary film. The thus corrected amount of calcium loss was used as a measure of HAp erosion.

Collection of Saliva, Storage and Analysis

Twenty minutes after removal of the HAp and porcelain discs from the mouth unstimulated and stimulated whole saliva was collected for a series of analyses. Unstimulated saliva was collected by the draining method in a preweighed plastic cup [Navazesh and Christensen, 1982]. Stimulated saliva was collected by chewing on a piece of Parafilm (Parafilm M, Pechiney Plastic Packaging Company, Chicago, Ill., USA) at a chewing frequency of 70 chews per minute during collection. After each collection period the plastic cup was reweighed and the salivary flow rate (ml/min) was estimated by dividing the volume of the saliva sample (1 g of saliva equals 1 ml) by collection time (min) [Navazesh and Christensen, 1982]. Immediately after collection the salivary pH of both unstimulated and stimulated saliva was measured using a calibrated glass pH electrode (Radiometer, PHM 84 Research Meter, G202C, Copenhagen, Denmark). Calibration was performed daily using standard buffers, pH 7.01 and 4.00 (measurement uncertainty for both \pm 0.015 units; Merck KGaA, Darmstadt, Germany). The buffering capacity was measured by adding 0.5 ml of 5 mM HCl to the saliva used for pH measurement. The final pH after addition of HCl was regarded as an indication of the buffering capacity of the saliva. The remaining saliva was transferred to Eppendorf tubes (Eppendorf AG, Hamburg Ger-

many) and centrifuged for 5 min at 10,000 g at 4°C [Silletti et al., 2007]. After centrifuging, the saliva supernatant was decanted and frozen in liquid nitrogen and stored at -80°C in plastic containers (Cryogenic Vials Nalgene tubes, Nalgene Nunc, Rochester, N.Y., USA).

Unstimulated and stimulated whole saliva was analysed for electrolytes (calcium, phosphorus, sodium, chloride) and urea concentration, total protein concentration and albumin. Sodium and chloride concentrations were measured after appropriate dilution using an ion-selective electrode. Total calcium was determined by a colorimetric assay based on the reaction of calcium with *o*-cresolphthalein complexone (Sigma-Aldrich, St. Louis, Mo., USA) in alkaline solution [Gindler and King, 1972]. Phosphorus concentration was measured by a modified acid-molybdate method [Chen et al., 1956]. A kinetic UV assay based on the method of Talke and Schubert [1965] was used to measure urea concentration. The total protein concentration was determined turbidimetrically. For this method the saliva sample was preincubated in an alkaline solution containing EDTA, which denatured the protein and eliminated interferences from ions. Benzethonium chloride was added to produce turbidity, which was measured at a wavelength of 505 nm [Luxton et al., 1989]. The albumin concentration was determined with an immunoturbidimetric assay. Anti-albumin antibodies were added to the saliva sample to form antigen/antibody complexes which, following agglutination, were measured turbidimetrically [Hubbuck, 1991]. All the above-mentioned analyses were performed on a Roche/Hitachi 911 analyser and a COBAS Integra Chemistry Platform (Roche Diagnostics, Indianapolis, Ind., USA).

Statistical Methods

Pearson's correlation coefficient analysis was used to study the association between HAp erosion and various salivary parameters. A *p* value of 0.05 or lower was considered statistically significant. Furthermore, multiple regression analysis with backward elimination was performed to determine the contribution of every saliva parameter to HAp erosion. This information was used to design a model containing all the variables of interest by a stepwise removal of the variable with the smallest *F* statistic (cut-off level for *p* to remain in the model: 0.1). For the explanation of the variation in HAp erosion by the model the adjusted r^2 was determined because it adjusts for the number of explanatory terms in a model. This was performed by R statistical software (version 2.10.1, R Development Core Team, 2009).

Results

Calcium loss from HAp (uncorrected) and from the pellicle plus salivary film is depicted in figure 1, showing individual variation. When relating the measured HAp erosion to the various salivary parameters, significant correlations were found between HAp erosion and the concentration of phosphorus in unstimulated saliva ($r = 0.40$, $p = 0.03$). For stimulated saliva a significant correlation was found between HAp erosion and the concentration of sodium ($r = -0.40$, $p = 0.03$), chloride ($r = -0.47$,

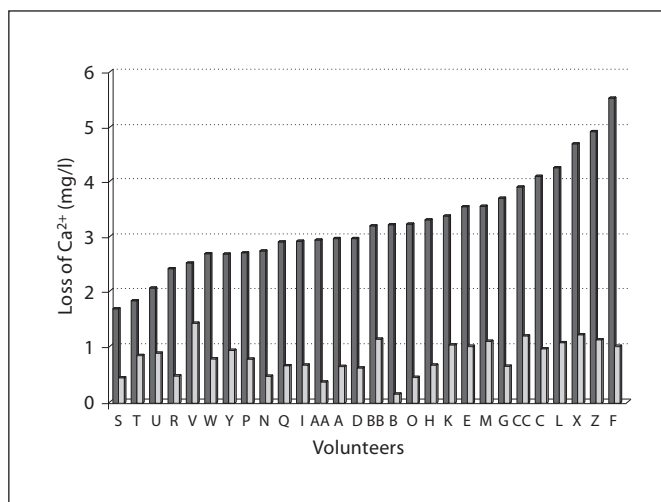


Fig. 1. Loss of calcium from HAp (black) and pellicle (grey) for all volunteers.

$p = 0.01$), phosphorus ($r = 0.45$, $p = 0.01$) and flow rate ($r = -0.39$, $p = 0.04$). All the correlation coefficients and the corresponding confidence intervals are presented in table 1. The results of the multiple regression analysis with backward elimination revealed that a significant role in HAp erosion was found for sodium, urea, total protein, albumin, pH and flow of unstimulated saliva and for sodium, potassium, urea, and phosphorus of stimulated saliva. From this data a model predicting HAp erosion was suggested as shown in table 2. Using these parameters 72% (adjusted $r^2 = 0.72$) of the variation in HAp erosion could be explained.

Discussion

This study investigated the relationship between whole saliva parameters and erosion of HAp. Analysis of the results revealed that several salivary parameters were related to the extent of erosion.

A higher flow rate of stimulated saliva was associated with a suppression of HAp erosion. This observation corresponded with earlier reports in which also an inverse relationship between stimulated salivary flow rate and erosion was shown [Jensdottir et al., 2005]. It was demonstrated that a high salivary flow rate resulted in higher concentrations of specific ions (such as sodium, calcium, chloride and bicarbonate) and proteins and was associated with a higher salivary buffer capacity [Larsen and Pearce, 2003; Dawes and Kubieniec, 2004]. Moreover, a

Table 1. Results of the analyses of (un)stimulated saliva, Pearson's correlation coefficients between HAp erosion and salivary parameters and 95% CI

Salivary parameter	Unstimulated saliva				Stimulated saliva			
	mean \pm SD	Pearson's correlation coefficient	p value	95% CI	mean \pm SD	Pearson's correlation coefficient	p value	95% CI
Sodium, mmol/l	4.00 \pm 1.79	-0.338	0.073	-0.63 to 0.03	11.47 \pm 7.72	-0.402	0.031*	-0.67 to -0.04
Potassium, mmol/l	23.61 \pm 5.20	-0.026	0.893	-0.39 to 0.34	21.05 \pm 4.20	-0.053	0.785	-0.41 to 0.32
Chloride, mmol/l	19.79 \pm 5.65	-0.020	0.916	-0.38 to 0.35	20.79 \pm 5.46	-0.472	0.010*	-0.72 to -0.13
Urea, mmol/l	6.89 \pm 2.60	0.154	0.424	-0.23 to 0.49	4.32 \pm 1.20	0.161	0.404	-0.22 to 0.50
Calcium, mmol/l	1.36 \pm 0.33	0.224	0.243	-0.16 to 0.55	1.05 \pm 0.20	0.082	0.672	-0.29 to 0.44
Phosphate, mmol/l	6.75 \pm 1.94	0.399	0.032*	0.04 to 0.67	4.50 \pm 1.25	0.450	0.014*	0.10 to 0.70
Total protein, g/l	0.34 \pm 0.22	-0.076	0.694	-0.43 to 0.30	0.29 \pm 0.17	-0.191	0.320	-0.52 to 0.19
Albumin, mg/l	30.33 \pm 19.30	0.268	0.160	-0.11 to 0.58	21.12 \pm 14.26	0.250	0.190	-0.13 to 0.57
pH	7.08 \pm 0.36	-0.118	0.543	-0.46 to 0.26	7.42 \pm 0.25	-0.056	0.773	-0.41 to 0.32
Buffer capacity ¹	1.10 \pm 0.44	-0.214	0.265	-0.54 to 0.17	0.90 \pm 0.25	-0.002	0.990	-0.37 to 0.36
Flow, ml/min	0.45 \pm 0.24	0.124	0.521	-0.25 to 0.47	1.93 \pm 1.01	-0.388	0.037*	-0.66 to -0.03

¹ pH after addition of 0.5 ml 5 mM hydrochloric acid to 0.5 ml saliva. *p \leq 0.05.

Table 2. Results from the multiple regression analysis with backward elimination (cut-off level \leq 0.1)

Variable	Type of saliva	Effect	p	95% CI
Constant		-11.40	0.002	-17.79 to -5.00
Sodium	unstimulated	-0.17	0.008	-0.29 to -0.05
Sodium	stimulated	0.07	0.010	0.02 to 0.12
Potassium	stimulated	-0.10	0.025	-0.19 to -0.01
Urea	unstimulated	-0.18	0.052	-0.36 to 0.00
Urea	stimulated	0.35	0.024	0.05 to 0.65
Phosphorus	stimulated	0.90	0.000	0.61 to 1.19
Total protein	unstimulated	4.68	0.004	1.67 to 7.69
Total protein	stimulated	-4.56	0.002	-7.15 to -1.97
Albumin	unstimulated	0.03	0.000	0.02 to 0.04
pH	unstimulated	1.39	0.003	0.54 to 2.23
Flow	unstimulated	1.62	0.000	0.84 to 2.40

high salivary flow rate resulted in a better clearance of acids from the tooth surfaces [Järvinen et al., 1991; Bashir et al., 1995]. In our model, clearance of acids from HAp surfaces cannot have played an important role as the HAp samples were extra-orally exposed to acids.

The concentration of chloride and sodium in stimulated saliva was found to be associated with the suppression of HAp dissolution as well. Earlier research has shown, however, that HAp dissolution is not inhibited by the incorporation of Cl⁻ ions into HAp through either ion

exchange or adsorption in an ambient aqueous solution [Sugiyama et al., 1999]. Therefore, it was suggested that the inhibition of dissolution of HAp by Na⁺ and Cl⁻ could be the result of a competition for HAp surface protonation sites between Na⁺ and H⁺ ions [Kwon et al., 2009]. In addition, it should be noted that Na⁺ and Cl⁻ ions account for more than 60% of the ionic strength of saliva [Schneyer et al., 1972] and therefore possibly may play a role in the dissolution process of HAp surfaces. Furthermore, the observed correlations could also be the result of an indirect effect as a rise in salivary flow rate is accompanied by a rise in sodium and chloride concentration.

Another electrolyte influencing the dissolution of HAp in our model was phosphorus in unstimulated and in stimulated saliva. It is suggested in earlier research that an increase in salivary phosphate concentration may result in desorption of salivary proteins from HAp [McGaughey and Stowell, 1974]. This effect could be important in our study in which the HAp was exposed to an acidic challenge in the presence of only the salivary pellicle. Higher phosphate concentrations in saliva result in desorption of proteins from HAp, which in turn result in a reduction of the protective strength of the salivary pellicle to an acidic challenge, increasing HAp erosion. Furthermore, it is shown that the phosphate concentration in saliva is inversely related to flow rate [Dawes and Kubieniec, 2004]. We showed that a high flow of saliva is asso-

ciated with lower HAp erosion. Therefore, the role of phosphate in HAp erosion could be an indirect effect of the flow. Some studies have shown that high susceptibility to erosion is associated with a low buffering capacity of saliva [Meurman et al., 1994; Lussi and Schaffner, 2000]. This was not confirmed in our study. In the extra-oral erosion model the effect of salivary buffer capacity on the loss of HAp was probably limited due to the small amount of saliva present on the HAp during the acidic challenge. Moreover, our method of collection of saliva and the determination of its pH and buffer capacity could have influenced the results. During collection of the saliva and determination of pH and buffer capacity, the saliva is exposed to the atmosphere causing a loss of CO₂. This loss of CO₂ causes a pH change in the alkaline direction, influencing the buffer capacity measurements [Bardow et al., 2000].

The experimental model with an extra-oral challenge concentrated on the effects of the pellicle and the adhering saliva film, and therefore does not incorporate the full potential of saliva in erosion protection, as mentioned before for flow and buffering capacity. Therefore it is surprising that flow rate was a significant factor in our study. Possibly, flow rate is related to a compositional factor that has not been measured yet.

For this study we used synthetically prepared HAp discs, which is a close analogue to human enamel mineral. The discs have greater porosity and their structure, particle size and shape differ from human enamel [Hemingway et al., 2008]. Due to the greater porosity of the HAp discs the absorption of proteins and especially of peptides may be higher compared to human enamel. HAp was used in many in vitro and in situ studies [Vacca Smith and Bowen, 2000; Barbour et al., 2008; Hemingway et al., 2008; Zaman et al., 2010]. The composition of HAp discs derived from the same batch is stable. This reduces variation in sample composition, making interindividual comparison of the saliva/pellicle effect more straightforward.

The pellicle's protective effect is lost within 10 min of exposure with a citric acid solution at pH = 2.3 [Nekrashevych and Stösser, 2003]. This model aimed at studying early erosion, using a shorter and milder erosive challenge, simulating a short period of drinking a citric acid-based drink with an intermediate pH [Jäger et al., 2008]. Using a porcelain disc control made it possible to correct the calcium loss measured for the HAp, for calcium lost from the pellicle/saliva film. Figure 1 shows that calcium loss from the pellicle/saliva film was significant and also showed considerable interindividual variation.

The multivariate model should be interpreted with considerable caution, as the balance between the variables that were studied and the volume of data is not ideal. It has been included only to give an indication of the variable most likely involved in the complex process. As the factors included are now corrected for all other factors included, a complex picture emerges, where saliva components appear to have opposing effects, depending on their source from stimulated or unstimulated saliva. The exact effect sizes are of little consequence. However, the model gives information about which salivary parameters, in addition to the ones appearing in the univariate analysis, could be of potential interest for further research.

Within the limits of this preliminary in vitro study it can be concluded that there are associations between several investigated salivary parameters and loss of pellicle/saliva-covered HAp due to an erosive challenge. Direct investigation of the pellicle itself, and its composition in relation to early erosion is needed to further clarify the protective role of various factors. Additionally, clinical research is needed to investigate whether or not these factors can be shown to play a role in clinical erosion and erosive wear.

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Disclosure Statement

There is no conflict of interest for any of the authors of this article that might introduce bias or affect their judgement.

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